OFFICE OF NAVAL RESEARCH

GRANT N00014-91-J-1784

R&T CODE 313v002

Technical Report No. 8

COLOCALIZATION OF CALCIUM ENTRY AND EXOCYTOTIC RELEASE SITES IN ADRENAL CHROMAFFIN CELLS

by

Iain M. Robinson, Jennifer M. Finnegan, Jonathan R. Monck, R. Mark Wightman and Julio M. Fernandez

Prepared for Publication in the

Proceedings of the National Academy of Science

Department of Chemistry
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-3290

January 13, 1995

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 13, 1995	TECHNICAL 10/94 - 1/95

4. TITLE AND SUBTITLE
COLOCALIZATION OF CALCIUM ENTRY AND EXOCYTOTIC RELEASE SITES IN ADRENAL CHROMAFFIN CELLS

5. FUNDING NUMBERS
N-00014-91-J-1784

6. AUTHOR(S)
Iain M. Robinson, Jennifer M. Finnegan, Jonathan R. Monck, R. Mark Wightman and Julio M. Fernandez

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Chemistry, CB # 3290
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-3290

8. PERFORMING ORGANIZATION REPORT NUMBER
ONR Technical Report VIII

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5000

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
submitted to the Proceedings of the National Academy of Science

12a. DISTRIBUTION/AVAILABILITY STATEMENT
This document has been approved for public release and sale; its distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

"Snapshot" images of localized Ca^{2+} influx into patch-clamped chromaffin cells were captured using a recently developed pulsed-laser imaging system. Transient opening of voltage-sensitive Ca^{2+} channels gave rise to localized elevations of Ca^{2+} that had the appearance of either "hotspots" or partial rings found immediately beneath the plasma membrane. When the Ca^{2+} imaging technique was employed in conjuntion with flame-etched crabon-fiber electrodes to spatially map the release sites of catecholamines, it was observed that the sites of Ca^{2+} entry and catecholamine release were colocalized. These results provide functional support for the idea that secretion occurs from "active zone"-like structures in neuroendocrine cells.

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	

5-Dec-94

Biological Sciences: Biophysics

Colocalization of Calcium Entry and Exocytotic Release Sites in Adrenal Chromaffin Cells.

(Pulsed laser Ca^{2+} imaging / amperometry / secretion / Active zones)

Iain M. Robinson, Jennifer M. Finnegan*, Jonathan R. Monck, R. Mark Wightman* and Julio M. Fernandez.

Department of Physiology and Biophysics,
Mayo Clinic,
Rochester, MN.
55905.

*Department of Chemistry,
University of North Carolina,
Chapel Hill, NC.
27599.

Accesion For	
NTIS CRA&I	X
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

Address correspondence to:- Dr. Julio M. Fernandez,
Department of Physiology and Biophysics,
1-117 Medical Sciences Building,
Mayo Clinic,
Rochester, MN.
55905. USA.
Fax: + 507 284 0521

Abstract.

"Snapshot" images of localized Ca^{2+} influx into patch-clamped chromaffin cells were captured using a recently developed pulsed-laser imaging system. Transient opening of voltage-sensitive Ca^{2+} channels gave rise to localized elevations of Ca^{2+} that had the appearance of either "hotspots" or partial rings found immediately beneath the plasma membrane. When the Ca^{2+} imaging technique was employed in conjunction with flame-etched carbon-fiber electrodes to spatially map the release sites of catecholamines, it was observed that the sites of Ca^{2+} entry and catecholamine release were colocalized. These results provide functional support for the idea that secretion occurs from "active zone"-like structures in neuroendocrine cells.

Introduction.

Neurons contain specialized regions at the plasma membrane where secretory vesicles accumulate. These regions, called "active zones", were first observed by electron microscopy (1,2). These active zones are also believed to contain clusters of voltage-sensitive Ca^{2+} channels (3). More recently, localized zones of Ca^{2+} entry, termed "hotspots", have been identified in neurons, both on dendrites (4,5) and at nerve terminals (6), and also in hair cells (7). Furthermore, it has been demonstrated that vesicular secretion is triggered by specific modes of Ca^{2+} influx across the plasma membrane (8-10). This raises the hypothesis that these sites of Ca^{2+} entry are colocalized at the plasma membrane with sites where vesicles are poised for fusion. Indeed a standard text book notion describes just such a scenario for neuronal cells, however there is no direct functional evidence to support this point. There have been no reports of morphological structures similar to the active zones found in neurons being present in neuroendocrine cells.

A recent report (11) has described the existence of hotspots of localized Ca^{2+} entry in patch-clamped bovine adrenal chromaffin cells following the transient opening of voltage-sensitive Ca^{2+} channels. The pulsed-laser imaging system employed in these studies has an improved spatial and temporal resolution compared with those imaging systems that have previously been used in attempts to capture the early stages of influx of Ca^{2+} into chromaffin cells. The approach adopted is similar to that used in "flash photography" where a brief transient illumination is used to freeze and capture an image. A pulsed-laser is used to provide a brief (350ns), high intensity (0.25J) epi-illumination to transiently excite the fluorescent Ca^{2+} indicator dye rhod-2 enabling the capture

of "snapshot" images of the intracellular Ca^{2+} concentration. Wightman and colleagues have independently developed carbon-fiber ultramicroelectrodes that are capable of monitoring secretion from areas of $2\mu m^2$ and have used these fibers to show that catecholamine release occurs from restricted zones in chromaffin cells (12). In this present study we have combined the pulsed-laser imaging system to resolve the intracellular Ca^{2+} distribution and the carbon-fiber micro-electrodes to measure, and spatially map, catecholamine release from individual vesicles. We demonstrate here, by combining the two techniques, that the sites of Ca^{2+} entry are indeed colocalized with active zones of catecholamine release in bovine adrenal chromaffin cells. Thus, for the first time the spatial and temporal proximity of the trigger for exocytosis, i.e. Ca^{2+} entry, and release are revealed. These results suggest that chromaffin cells contain structures that are functionally akin to the active zones found in neuronal cells.

Materials and Methods.

Cell Preparation and Culture.

Chromaffin cells were prepared from bovine adrenal medullae by enzymatic digestion (13). Isolated cells were resuspended in Dulbecco's modified Eagles medium (DMEM) supplemented with 25mM HEPES, 10% fetal calf serum, 8μM fluorodeoxyuridine, 50μg/ml gentamycin, 10μM cytosine arabinofuranoside, 2.5μg/ml fungizone, 25U/ml penicillin and 25μg/ml streptomycin and plated at a density of 100,000 cells/ml on glass coverslips in 35mm diameter petri dishes. Cells were cultured in a humidified atmosphere at 37°C in the presence of 5% CO_2 for 1-5 days prior to use. For experiments, cells were washed in an extracellular medium comprising 120mM NaCl, 20mM HEPES, 4mM $MgCl_2$, 2mM

$CaCl_2$, 4mg/ml glucose and 1μM tetrodotoxin at pH 7.2 (NaOH). The patch pipette solution contained either 120mM Cs-D-glutamate, 30 mM HEPES, 8mM NaCl, 1mM $MgCl_2$, 2mM ATP, 0.3mM GTP and 100μM EGTA, or for imaging studies 120mM Cs-D-glutamate, 30 mM HEPES, 8mM NaCl, 1mM $MgCl_2$, 2mM ATP, 0.3mM GTP and 0.4mM rhod-2 (triammonium salt) (pH 7.2). Experiments were carried out at room temperature.

Measurement of Membrane Capacitance.

In some cases we monitored exocytosis by measuring the cell membrane capacitance, using the whole-cell mode of the patch-clamp technique (14), in conjunction with a digital phase detector (15) implemented on a system comprising an IDA data acquisition interface (Indec Systems, Sunnyvale, CA) and an EPC-7 patch clamp amplifier (List Electronics, Darmstadt, Germany). A sinusoidal voltage (833Hz, 54mV peak to peak) on top of a holding potential of -60mV was applied to the patch-clamped cell, and the current was measured at two different phase angles, ϕ and ϕ-π/2, relative to the stimulus. The phase was periodically adjusted using the phase tracking technique (16), so that the output at ϕ reflected changes in the real part of the cell admittance (Re [ΔY]), whereas the output at ϕ-π/2 reflected changes in the imaginary part of the admittance (Im [ΔY]), which was used as a measure of the cell membrane capacitance (16).

Fluorescence Imaging of Intracellular Ca^{2+} Concentrations.

The imaging system consisted of a Zeiss epifluorescence microscope, a Photometrics cooled CCD camera, and a Compaq 386 host computer to control image acquisition and image processing using a Mercury array processor. To this was added a high intensity pulsed coaxial flash lamp dye laser (Model LS-1400, Luminex) to provide short (350ns) high intensity pulses of illumination and

a patch clamp set up comprising a List EPC-7 patch-clamp amplifier and an Indec IDA15125 interface for data acquisition and synchronization of the laser with the depolarization stimulus. The laser was coupled with a liquid light guide to the epi-fluorescence port of the microscope and used to illuminate the entire field of view. The lasing dye used was Coumarin 521 (0.02mM in methanol) which gave a suitable emission spectrum for excitation of rhod-2. The epifluorescence filter block contained a 570nm DRLP dichroic mirror and a 585nm EFLP emission filter (Omega Optical, Brattleboro,VT). A Zeiss 40x objective (0.65 N.A.) was used to image the cells. Image pairs were taken for control and depolarizing stimulus pulses (11,17). The images were filtered for noise reduction (11). The ratio of the stimulus image divided by the control image is displayed as a pseudo-color image representing the fractional change in fluorescence. Since the ratio corrects for differences in indicator concentration, indicator excluded volume and light path length, the fractional change in fluorescence represents a measure of Ca^{2+} change provided that the cell does not move between the control and stimulus image. Previous estimates put the observed Ca^{2+} changes in the region of 200-300nM assuming a resting intracellular Ca^{2+} concentration of 100nM (11).

Amperometric detection of catecholamine secretion.

In these studies two types of carbon-fiber electrodes were used to monitor the release of catecholamines, one type was large, having tip diameters similar to those of the cell (~14μm), whereas those used to map the release sites had tip diameters of ~2μm. In both cases fabrication of the electrodes required the aspiration of a carbon-fiber into a capillary pipette which was then pulled on a micropipette puller. In the case of the larger electrodes (those used in fig. 1), the carbon-fiber was sealed to the glass capillary tube by immersing the tips of the

fibers into epoxy. This was cured overnight at room temperature, then at 100 • C for 2 hrs., and finally at 150 • C for a further 2 hrs. (18). Before use, the tip of the fiber was beveled at 45° using a micropipette beveler resulting in an elliptically shaped carbon-fiber tip. The carbon electrodes used to spatially map the release of catecholamines (used in fig. 2) were constructed in a slightly different manner. After pulling the glass pipette, approximately 500µm of the carbon-fiber was exposed beyond the end of the glass capillary tube and was etched in a Bunsen burner flame resulting in the formation of a carbon cone with a submicron tip. The pipettes were then filled with colloidal graphite into which a chromel wire was inserted to establish an electrical connection. The fibers were then insulated with poly(oxyphenylene) (~1µm thick) by electrochemical deposition for 8 mins. at +4V, and cured for 40 mins. at 150°C (12,19,20). The tip of the fiber was beveled at an angle of 45° for 5s to remove the insulating layer at the very tip of the electrode using a micropipette beveler. Both types of electrode were held at a voltage of +650mV and the redox current was monitored with an Axopatch 1B patch-clamp amplifier; at this voltage, catecholamines are the main substances released from chromaffin granules that will be oxidized.

Results and Discussion.

Amperometry is a powerful technique for monitoring the release of catecholamines from chromaffin cells. Single amperometric "spikes" have been attributed to the release of a single vesicle's contents (21). Figure 1a shows the amperometric recording obtained following the brief depolarization (50 ms) to +20mV of a single patch-clamped adrenal chromaffin cell held at -60mV. Several spikes (superimposed upon one another) were detected suggesting that several vesicles fused with the plasma membrane. This view is supported by the

simultaneous measurement of cell membrane capacitance which showed a 24fF step increase after the depolarizing pulse corresponding to the fusion of 2-24 vesicles (1a). Randomly occurring single fusion events were often observed; two examples of such events are shown in fig. 1b and c. In both cases each step in membrane capacitance is accompanied by only a single spike, corresponding to the release of the contents of a single vesicle. In fig. 1b, a foot can be seen that precedes the main spike (22,23), in contrast there is no observable foot in the event seen in fig. 1c. The simultaneous capacitance measurement shows that the foot develops immediately after fusion began. These results are similar to those reported in mast cells where the foot was shown to be due to i) the fusion pore that forms between secretory vesicles and the plasma membrane and ii) the manner in which the vesicle's contents are released from a polymer gel matrix that fills the inside of the secretory vesicle (23-25). Similar vesicular association has been inferred in release studies from chromaffin cells (26, 27). The measurements shown in fig.1 only report the extent of secretory excursions from the entirety of the plasma membrane and provide no spatial data as to where the events took place. In order to answer this question, we combined pulsed-laser Ca^{2+} imaging with flame-etched carbon-fiber microelectrodes to spatially map the release sites of catecholamines in chromaffin cells.

Figures 2a and d show "snapshot" images of changes in intracellular Ca^{2+} concentration in single patch clamped bovine adrenal chromaffin cells obtained with a pulsed-laser imaging system. This imaging technique (11,17,28) relies on the transient high intensity illumination of a pulsed-laser (350 ns duration) to excite fluorescent Ca^{2+} indicator molecules (11,17). Non-inactivating Ca^{2+} currents were induced by depolarizing patch-clamped chromaffin cells (in the whole-cell recording mode) from a holding potential of -60mV to +20mV for

50ms. The laser pulse was synchronized to fire 5 ms after the end of the depolarizing pulse. In some instances, discrete increases in cytosolic Ca^{2+} levels that had the appearance of "hotspots" were observed (fig. 2a and d), whereas in other cells these hotspots had merged into partial rings (data not shown). These differences in the patterns of localized Ca^{2+} influx were seen from cell to cell, however the pattern in each cell was reproducible (11). In all cases the largest increases in intracellular Ca^{2+} concentration were seen immediately beneath the plasma membrane (fig. 2a and d). Depolarizations lasting longer than 100 ms resulted in a loss of the hotspot pattern and a ring-like distribution of Ca^{2+} filling the cytosol beneath the plasma membrane (not shown). Previous imaging studies using more conventional imaging techniques reported that the effect of depolarizing stimuli on the influx of Ca^{2+} into adrenal chromaffin cells resulted in the elevation of Ca^{2+} as a ring under the plasma membrane (29,30). The comparatively slow temporal resolution of these systems versus the pulsed-laser imaging system used in this study would account for the fact that "hotspots" of Ca^{2+} influx have not been described in earlier studies in chromaffin cells. These localized changes in intracellular calcium observed during brief depolarizations ($\leq$ 50 ms) are proposed to represent Ca^{2+} entry through clusters of Ca^{2+} channels in the plasma membrane (11).

Evidence for spatially localized secretion from both cultured (12,31-33) and in vivo (34) chromaffin cells has been reported. Schroeder et al. (12) used flame-etched carbon-fiber microelectrodes which detect the release of catecholamines from areas of only 1-2μm^2, to demonstrate this point. In these experiments isolated bovine adrenal chromaffin cells were stimulated to secrete with prolonged exposure to either barium, potassium or nicotine in the presence of extracellular Ca^{2+}. These conditions cause an initial increase in the intracellular

Ca^{2+} concentration immediately beneath the plasma membrane. At longer times Ca^{2+} diffuses throughout the cell, resulting in a sustained and homogeneous increase in cytosolic calcium (35). Thus the results of Schroeder et al. (12) suggest that secretion remains localized in discrete areas of the cell even under conditions which lead to global elevations of Ca^{2+}. Furthermore experiments carried out using intact chromaffin cells established that elevating intracellular Ca^{2+} using various agonists only led to appreciable amounts of secretion in the presence of extracellular Ca^{2+} (21,35). Recent reports suggested that the pathway of entry and not merely the magnitude of the change in the intracellular Ca^{2+} concentration can determine the extent of the secretory response. It has been shown that opening of different voltage-sensitive Ca^{2+} channels stimulates secretion to different degrees (8-10). These data suggests that there is an important spatial component of the Ca^{2+} signal underlying stimulus secretion coupling. This raised the interesting question of whether the Ca^{2+} "hotspots" observed here are colocalized to the zones of secretion described above.

By combining the pulsed-laser imaging technique and the measurement of local catecholamine secretion we demonstrate that this is indeed the case (see fig. 2 and table I). Fig. 2 shows pseudo-color images of the distribution of intracellular Ca^{2+} for two separate cells following a 50ms depolarizing stimulus (fig. 2a and d). Bright field images of the cells depicted in fig. 2a and d are shown in fig. 2b and c, and, 2e and f respectively. The patch-pipette can clearly be seen on the right of the cell at the 3 o'clock position; in fig. 2b and e the flame-etched fiber can be seen to the left of the cell at 9 o'clock, and in fig. 2c and f it was moved to 7 o'clock and 11 o'clock respectively. In all cases the carbon-fiber electrodes were placed in close proximity to the plasma membrane (~0.6 µm (n=49)) and the distance from the center of the carbon-fiber electrode to the nearest hotspot

was measured. Immediately beneath the bright field images, amperometric recordings are shown that were obtained from the flame-etched electrodes shown in their respective positions above (figs. 2b, c, e and f). It can clearly be seen that when the fibers were moved to a position adjacent to a Ca^{2+} hotspot, a secretory response was observed (fig. 2 and Table I). In contrast, amperometric spikes were observed far less frequently when the fibers were placed in apposition to areas of the cell in which the intracellular Ca^{2+} levels had increased to a lesser extent (fig. 2 and Table I). Positioning of the amperometric fiber close to a Ca^{2+} hotspot ($1.4 \pm 0.2\mu m$ (n=38)) resulted in the detection of a secretory response 74% of the times that the cell was stimulated; this figure dropped to only 18% when the fiber was away from any hotspot ($3.6 \pm 0.4\mu m$ (n=19)) (Table I).

The results described above demonstrate that under conditions leading to discrete elevations of Ca^{2+}, secretion occurs from sites that have the functional characteristics of active zones. The next question that needs to be answered is how are these secretory zones organized and what are the roles played by various plasma membrane, vesicular and cytosolic proteins in establishing these sites in the cell? One scenario that has been envisaged involves the interaction of the vesicle bound Ca^{2+}-sensing protein synaptotagmin with an integral plasma membrane bound protein, syntaxin, that has been shown to interact with Ca^{2+} channels (36). Thus targeting of secretory vesicles to specific points on the plasma membrane could result in a clustering of Ca^{2+} channels, or, more simply that clusters of Ca^{2+} channels could represent the target sites for the docking of secretory vesicles in the first place. Alternatively, cysteine string proteins have been shown to be essential subunits or regulators of presynaptic calcium channels (37) and deletion of this protein in *Drosophila* was shown to inhibit

synaptic transmission (38). These proteins were expected to be plasma membrane associated proteins. Surprisingly, it was recently found that cysteine string proteins are found in the membrane of secretory vesicles of *Torpedo* electric organ (39). These results strongly suggest that a tight association between secretory vesicles and calcium channels is required for calcium channel function, restricting calcium influx to those sites where secretory vesicles are docked in a fusion ready state (39). Thus only Ca^{2+} channels associated with docked vesicles would open in response to a depolarizing pulse, providing a possible explanation for the colocalization of Ca^{2+} hotspots and release sites observed in chromaffin cells. The remarkable conservation of the exocytotic machinery (40) suggests that the organization of the catecholamine release sites revealed in this work may be directly applicable to understanding the molecular basis for the organization of the active zones in neurons.

Acknowledgment

This work was funded by grants from the National Institute of Health to Julio Fernandez and the Office of Naval Research to Mark Wightman. Jennifer Finnegan was supported by a Department of Education Predoctoral Fellowship.

References.

1. Couteaux, R., and Pécot-Dechavassine, M. (1970) *C.R. Acad. Sc. Paris, Sér. D* **271**, 2346-2349.
2. Heuser, J.E. (1989). *Quart. J. Exper. Physiol.* **74,** 1051-1069.
3. Pumplin, D.W., Reese, T.S., and Llinás, R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7210-7213.
4. Muller, W., and Connor, J.A. (1991) *Nature (Lond.)* **354,** 73-76.
5. Guthrie, P.B., Segal, M., and Kater, S.B. (1991) *Nature (Lond.)* **354,** 76-80.
6. Llinás, R., Sugimori, M., and Silver, R.B. (1992) *Science (Wash. D.C.)* **256,** 677-679.
7. Issa, N.P. and Hudspeth, A.J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10362-10366.
8. Hirning, L.D., Fox, A.P., McCleskey, E.W., Olivera, B.M., Thayer, S.A., Miller, R.J., and Tsien, R.W. (1988) *Science (Wash. D.C.)* **239,** 57-61.
9. Wheeler, D.B., Randall, A., and Tsien, R.W. (1994) *Science (Wash. D.C.)* **264,** 107-111.
10. Artalejo, C.R., Adams, M.E., and Fox, A.P. (1994) *Nature (Lond.)* **367,** 72-76.
11. Monck, J.R., Robinson, I.M., Escobar, A., Vergara, J., and Fernandez, J.M. (1994) *Biophys. J.* **67**, 505-514.
12. Schroeder, T.J., Jankowski, J.A., Senyshyn, J., Holz, R.W., and Wightman, R.M. (1994) *J. Biol. Chem.* **269**, 17215-17220.
13. Burgoyne, R.D., Morgan, A., and O'Sullivan, A.J. (1988) *FEBS Lett.* **238,** 151-155.
14. Neher, E. and Marty, A. (1982) *Proc. Natl. Acad. Sci. USA.* **79**, 6712-6716.
15. Joshi, C. and Fernandez, J.M. (1988) *Biophys. J.* **53**, 885-892.

16. Fidler, N. and Fernandez, J.M. (1989) *Biophys. J.* **56**, 1153-1162.

17. Monck, J., Escobar, A., Robinson, I., Vergara, J. and Fernandez, J. (1994) *Biophys. J.* **61**, A351 (abstr.).

18. Kawagoe, K.T., Zimmerman, J.B. and Wightman, R.M. (1993) *J. Neurosci. Meth.* **48**, 225-240.

19. Kawagoe, K.T., Jankowski, J.A., and Wightman, R.M. (1991) *Anal. Chem.* **63**, 1589-1594.

20. Strein, T.G. and Ewing, A.G. (1992) *Anal. Chem.* **64**, 1368-1373.

21. Wightman, R.M., Jankowski, J.A., Kennedy, R.T., Kawagoe,K.T., Schroder, T.J., Leszczyszyn, D.J., Near, J.A., Diliberto, Jr., and Viveros, O.H. (1991) *Proc. Natl. Acad. Sci.* **88,** 10754-10758.

22. Chow, R.H. and Neher, E. (1992) *Nature (Lond.).* **356**, 60-63.

23. de Toledo, G.A., Fernandez-Chacon, R. and Fernandez, J.M. (1993) *Nature (Lond.).* **363**, 554-557.

24. Monck, J.R. and Fernandez, J.M. (1994) *Neuron.* **12**, 707-712.

25. Robinson, I.M. and Fernandez, J.M. (1994) *Curr. Opp. Neurobiol.* **4**,330-336.

26. Jankowski, J.A., Schroeder, T.J., Ciolkowski, E.L. and Wightman, R.M. (1993) *J.Biol.Chem.* **268**, 14694-14700.

27. Jankowski, J.A., Finnegan J.M. and Wightman, R.M. (1994) *J.Neurochem.* **63**, 1739-1747.

28. Kinosita, K., Jr., Ashikawa, I., Hibino, M., Shigemori, M., Yoshimura, H., Itoh, H., Nagayama, K., and Ikegami, A. (1988) *SPIE.* **909,** 271-277.

29. Cheek, T.R., O'Sullivan, A.J., Moreton, R.B. Berridge, M.J., and Burgoyne, R.D. (1989) *FEBS Lett.* **247,** 429-434.

30. Neher, E., and Augustine, G.J. (1992) *J. Physiol. (Lond.)* **450,** 273-301.

31. Phillips, J.H., Burridge, K., Wilson, S.P., and Kirshner, N. (1983) *J. Cell Biol.* **97,** 1906-1917.

32. Hesketh, J.E., Ciesielski-Treska, J., and Aunis, D. (1981) *Cell and Tiss. Res.* **218,** 331-343.

33. Cheek, T.R., Jackson, T.R., O'Sullivan, J.A., Moreton, R.B., Berridge, M.J., and Burgoyne, R.D. (1989) *J. Cell. Biol.* **109,**1219-1227.

34. Carmichael, S.W., Brooks, J.C., Malhotra, R.K., Wakade, T.E., and Wakade, A.R. (1989) *J. Electron Microscopy Tech.* **12,** 316-322.

35. Burgoyne, R.D. (1991) *Biochim. Biophys. Acta* **1071,** 174-202.

36. Bennet, M.K., Calakos, N., and Scheller, R.H. (1992) *Science (Wash. D.C.)* **257,** 255-259.

37. Gundersen, C.B., and Umbach, J.A. (1992) *Neuron* **9,** 527-537.

38. Mastrogiacomo, A., Parsons, S.M., Zampighi, G.A., Jenden, D.J., Umbach, J.A., and Gundersen, C.B. (1994) *Science (Wash. D.C.)* **263,** 981-982.

39. Zinsmaier, K.E., Eberle, K.K., Buchner, E., Walter, N., and Benzer, S. (1994) *Science (Wash. D.C.)* **263**, 977-980.

40. Bennet, M.K., and Scheller, R.H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2559-2563.

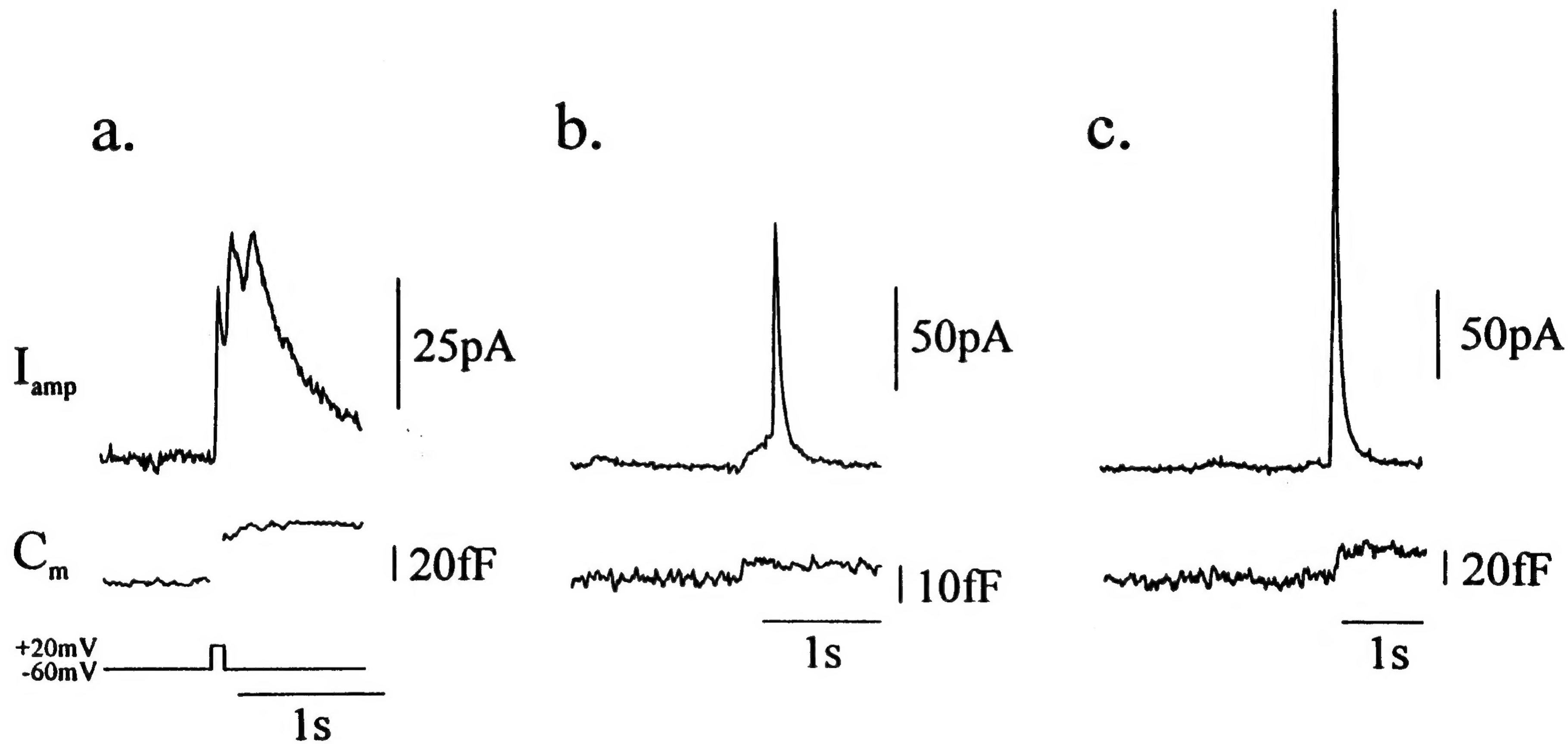
Figure Legends.

Figure 1. Secretory events monitored by simultaneous amperometric (I_{amp}) and capacitance (C_m) measurements demonstrate typical patterns of release. Fig. 1a shows a wide amperometric response composed of multiple spikes due to the fusion of several secretory vesicles triggered by a 50ms depolarization to +20mV from a holding potential of -60mV. From the magnitude of the capacitance response (24fF) we estimate that 2-24 vesicles fused with the plasma membrane. The exact number of vesicles that fused is not known since there is a distribution in their size. The amperometric response shows at least 3-5 discernible peaks that lag the capacitance step. However, the broad amperometric response (a) is likely to be composed of many more spikes. Two particularly large, isolated fusion events are shown in b and c. A clear lag between the fusion of a vesicle and the main spike of release can sometimes be observed (b), however, it is not always present (c). This foot contributes to make multiple fusion events, like those of 1a seem wider than those resulting from single vesicle fusion (compare a with b and c).

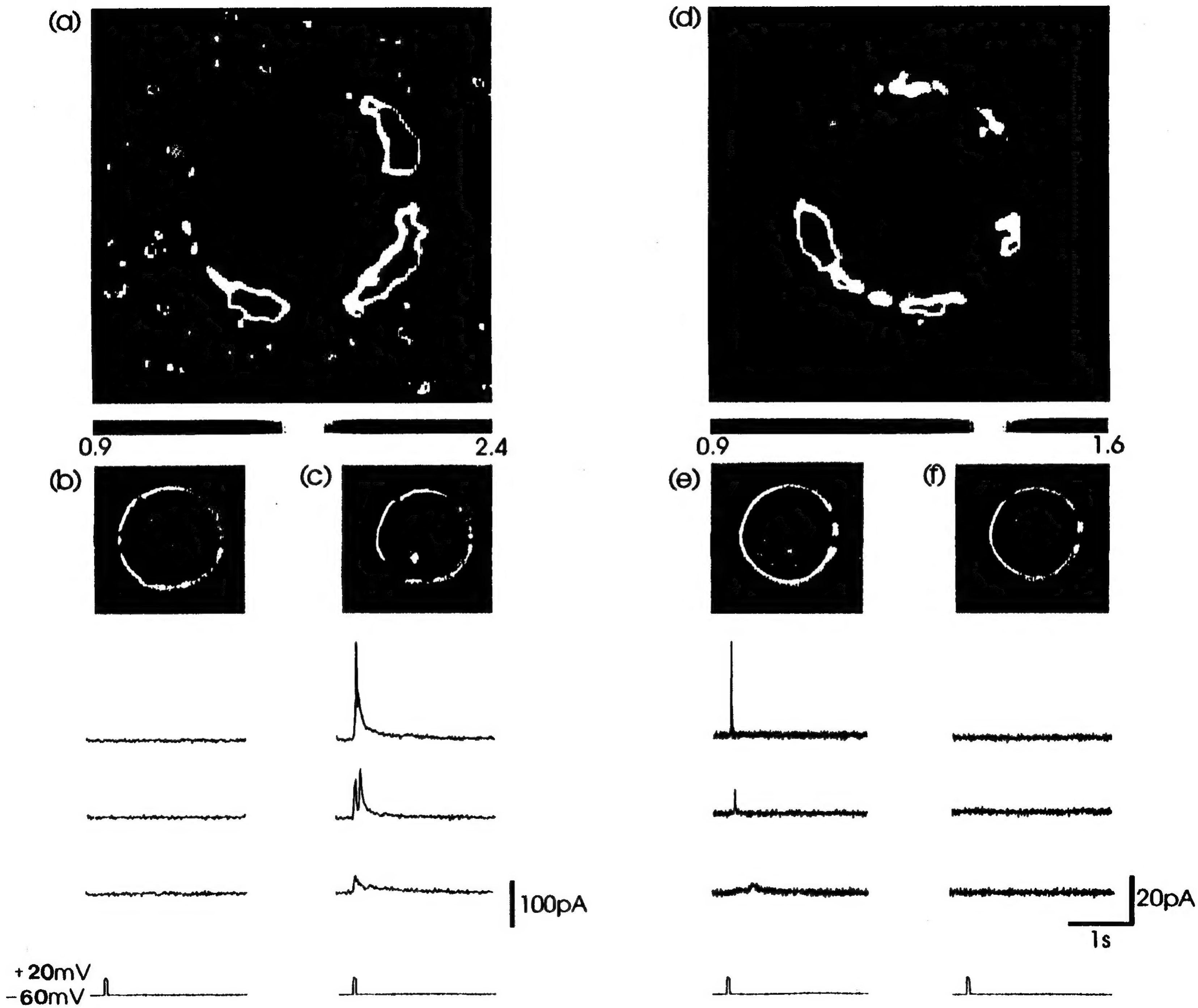
Figure 2. Ca^{2+} entry and secretion are colocalized in chromaffin cells. Submicrosecond "snapshots" of intracellular Ca^{2+} distribution reveal local "hotspots" of elevated Ca^{2+} in response to membrane depolarization. Images are ratios of control (no depolarizing pulse) and stimulus images taken 5ms after the end of a 50ms depolarizing pulse to +20mV from a holding potential of -60mV; images were taken 5s apart. The patch pipette is located at the 3 o'clock position in all cases. Discrete influxes of Ca^{2+} lead to the generation of "hotspots" of Ca^{2+} in some cells (a and d), whereas in other cells these hotspots are more

diffuse and can have the appearance of partial rings (data not shown). Mapping of the catecholamine release sites with flame-etched carbon-fiber microelectrodes revealed that these were colocalized with the areas of Ca^{2+} entry. A pair of bright field images are shown beneath each of the two Ca^{2+} images, (b) and (c) are of the cell shown in (a), and, (e) and (f) are of the cell shown in (d). The patch pipette can clearly be seen in all cases in the 3 o'clock position. In both cases the carbon-fiber electrode was initially positioned in the 9 o'clock position (b) and (e) (the Ca^{2+} images were obtained with the carbon-fibers in this position, where they did not obscure the cell). In the case of the cell on the left-hand side of the figure, the carbon-fiber electrode was positioned near, 1μm away from the plasma membrane, and 6μm away from the nearest Ca^{2+} "hotspot". In this position no amperometric signal was detected (b) (3 depolarization pulses); the carbon-fiber electrode was then moved to 1.5μm from the hotspot at the 7 o'clock position (c) resulting in the detection of an amperometric signal (3 of 3 depolarizing pulses). For the cell depicted in the right-hand side of the figure, a single Ca^{2+} "hotspot" at 9 o'clock (d) apposed the initial position of the carbon-fiber electrode (1.2μm from the tip of the electrode (the electrode was 0.9μm away from the plasma membrane)) (e) representative amperometric signals recorded from this position are shown immediately beneath the bright field image (e) (in this case 8 of 10 depolarizing pulses gave rise to amperometric spikes). Positioning the fiber at the 11 o'clock position (and virtually touching the plasma membrane), 6.2μm away from the nearest hotspot (f) resulted in a decrease in the number of spikes observed (only 2 of 10 pulses stimulated a secretory response). The depolarizing pulse protocol that was used for these experiments is shown at the bottom of the figure. (Note: each image is $17\mu m^2$.)

Table 1. Summary of the number of events seen in the amperometric recordings when the carbon-fiber electrode was either closely apposed to hotspots (1.4 ± 0.2μm (n=38)) (see fig. 2) or, when it was positioned next to areas in which the intracellular Ca^{2+} levels increased to a lesser extent (3.6 ± 0.4μm (n=19) away from the nearest hotspot). In all cases the carbon-fiber electrodes were placed in close proximity to the plasma membrane (~0.6 μm (n=49)) and the distance to the nearest hotspot was measured from the center of the carbon-fiber electrode. It is clear that positioning the carbon-fiber electrode close to the areas of Ca^{2+} influx resulted in the detection of a secretory response more frequently than when the fiber was away from the Ca^{2+} hotspot.



a.
b.
c.
I amp
C m
+20mV
-60mV
25pA
20fF
1s
50pA
10fF
1s
50pA
20fF
1s



(a)
(d)
0.9
2.4
0.9
1.6
(b)
(c)
(e)
(f)
100pA
20pA
1s
+20mV
-60mV

Near Hotspot			
Number of cells	Number of pulses	Number of spikes	% success rate
34	216	203	74

Away from Hotspot			
Number of cells	Number of pulses	Number of spikes	% success rate
24	160	33	18

A "success" was defined as the detection of amperometric spikes following stimulation of the cell with a depolarising pulse. The success rate is thus the number of successes ÷ total number of pulses x 100.

(Note that some depolarising pulses elicited more than one spike, but for the calculation of the % success rate these multiple spike events were only counted as single positives.)